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Trichinosis Flocculation Test

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Leonard A. Scheele, Surgeon General

Division of Public Health Methods
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Mouse-Protective Potency Assay of Typhoid Vaccine

—As Performed at the Army Medical Service Graduate School—

By H. C. Batson, Ph. D., Martha Brown, and Maurice Oberstein *

The need for an efficient, practical laboratory method for determining the potency of typhoid vaccines long has been recognized by immunologists concerned with the development, production, and assay of the product. The need in relation to production and control in routine manufacture of typhoid vaccine is mainly for a testing method which will permit the ready demonstration that an individual lot, or successive lots, of vaccine equal or exceed an established level of potency. Researchers, on the other hand, require an assay method which, in addition to the foregoing, permits quantitative estimation of potency, i. e., which permits numerical expression of potency in absolute or relative terms with an estimable degree of uncertainty. Only by the use of such a method is the investigator enabled to detect or evaluate differences or changes in potency as effected by experimental treatments or conditions of interest.

Methods early used in the determination of the potency of typhoid vaccines were based upon parenteral administration of the products to rabbits and subsequent *in vitro* determination of the agglutinin response (*15*), or the actual protection of such immunized rabbits against subacute infection following injection of living, virulent *Salmonella typhosa* (*18*). It is beyond the scope of this report to discuss or criticize these tests in detail and it is considered sufficient to state that such methods have proved to be neither adequately definitive nor reproducible to warrant their continued use.

Investigation and establishment by Siler and his associates at the Army Medical School (*22*) of the mouse-protection test for assessing the antibody response of humans to typhoid immunization stimulated interest in the adaptation of this technique to the assay of typhoid

*Director, Bacteriologist, and Research Assistant, respectively, Biologic Products Division, Army Medical Service Graduate School, Washington, D. C.

vaccines by means of active-immunity protection tests in mice. Although these workers did not employ this procedure as a control measure in relation to routine vaccine production, they did make extensive application of the method in evaluating experimental vaccines prepared from different strains of *S. typhosa* (*21*, *23*). Shortly thereafter, the National Institutes of Health adopted a similar mouse-protection potency test as official for release of commercially produced typhoid vaccine (*20*).

Relatively extensive studies on the mouse-protection test were conducted nearly simultaneously by Griffitts (*11*) and by Luippold (*17*). Methods proposed by these workers incorporated certain improvements in technique yet have failed to yield satisfactory reproducible quantitative results when employed in different laboratories or even in repeat tests conducted on the same product in the same laboratory.

In view of the continued need for a more reliable assay procedure, the Biologic Products Division, Army Medical Service Graduate School, has conducted an extensive study of the problem during the last 3 years. Phases of the study have dealt with the validity of certain assumptions regarding the nature of the immune response in mice, the validity and efficiency of assay procedure designs, the effects of associated or experimental conditions on quantitative results, the analysis and interpretation of assay results, etc. Results of these studies have been (*1*, *2*, *3*, *4*, *5*) or will be presented in detail and defended elsewhere. This report presents, essentially in didactic manner, the methods and procedures currently employed in these laboratories with the hope that such information will be of interest and aid to other workers responsible for conducting and interpreting potency assays on typhoid vaccine.

Materials and Methods

General Principles of the Assay Method

The potency assay of typhoid vaccine as currently performed in the Army Medical Service Graduate School is an active-immunity mouse-protection test in which the potency of an "unknown" vaccine is determined in relation to that of an arbitrarily selected reference standard vaccine.

The assay procedure involves the determination of the survival rates in successive groups of mice given graded doses of the vaccines and, subsequently, a constant challenge dose of viable *S. typhosa* suspended in 5-percent hog gastric mucin. In routine tests, only 2 doses or levels (referred to as low and high) of each, the unknown and the reference vaccine, are administered. In more exacting tests, in which it is wished to minimize the uncertainty of potency estimation, 3 or even 4 graded doses (levels) of each vaccine frequently are used.

The testing procedure, as presented here, is employed with either monovalent or triple typhoid vaccines. It has been the experience in these laboratories that such products apparently are of equal protective potency against challenge with *S. typhosa* provided they are of equal content of the specific organism. As yet no test has been developed for determining the protective potency of triple typhoid vaccine against *Salmonella paratyphi* (Para A) or *Salmonella schottmuelleri* (Para B).

The Reference Standard Vaccine

The particular vaccine currently used as a reference standard is a typical heat-killed phenol-preserved triple typhoid vaccine containing the equivalent of 1,000 million *S. typhosa* and 250 million each of *S. paratyphi* and *S. schottmuelleri* per ml. This vaccine was prepared in the same manner as are all lots of triple typhoid vaccine produced at the Army Medical Service Graduate School (*12*, *23*).[1]

A monovalent reference standard vaccine also has been prepared in a similar manner except for omission of the paratyphoid components. This vaccine is used as the reference in assays on experimental monovalent vaccines but, as stated previously, the data available fail to provide any evidence of a consistent difference in protective potency against *S. typhosa* challenge of the monovalent and triple typhoid reference vaccines.

The reference vaccines are maintained in both the lyophilized and the liquid states. The latter currently is employed in routine potency assays as extensive experience with these and similar vaccines has revealed no appreciable decrease in protective potency of either during a period of approximately 3 years.

In the assay procedure, an unknown lot of vaccine is compared directly with the reference vaccine in concomitant and identical tests. In practice, each of several unknown lots of vaccine frequently are compared with the same reference vaccine. Such tests on multiple unknowns are not truly independent, but the experience in these laboratories has not indicated this to be a major source of error.

Bacterial Content of Vaccines

Accurate determination of the potency of an unknown vaccine in relation to that of a standard (relative potency) is best accomplished when the bacterial content or density of the two are identical. Density determinations based upon photometric readings, or particularly upon direct counts, progressively decrease with aging of typhoid vaccines.

[1] In accordance with the assumptions upon which assays of this type are based, it is essential that the reference vaccine be prepared by a procedure identical with that employed in preparing the unknowns to be tested. Accordingly, this reference vaccine is suitable for use only in testing typhoid vaccines prepared by the heat-phenol method.

Such methods no doubt are sufficiently accurate for use in standardizing suspensions of freshly harvested organisms during production of vaccines, but it is considered that a more definitive quantitative determination is required for purposes of assay.

Preliminary experience with chemical determinations of the nitrogen content of typhoid vaccines has indicated that such data are sufficiently reproducible to serve as a basis for preliminary adjustment of samples to equal bacterial content. The method currently utilized in the determination of nitrogen content is that proposed by Simmons and Gentzkow (*24*) and involves micro-Kjeldahl digestion followed by nesslerization and comparison with prepared standards. The nitrogen content of the reference standard triple typhoid vaccine employed in this study has been determined as 0.041 mg. per ml. by this method. The nitrogen content of most routine lots of triple typhoid vaccine ranges from 0.040 to 0.045 mg. per ml.[2]

When necessary, unknown vaccines are adjusted by preliminary dilution with sterile normal saline to the same nitrogen content (± 10 percent) as that of the reference vaccine. When lyophilized reference vaccine is employed, the dried material is rehydrated to original (or stated) volume with sterile triple distilled water.

Vaccine Dilutions

Final dilutions of the reference vaccine (liquid or rehydrated) and of the unknown vaccines are prepared by dilution of appropriate volumes of each with sterile normal saline so that the desired individual quantities of vaccine are each contained in a unit volume of 0.5 ml.

The fold increments in quantities of vaccine administered at successive dosage levels depend, in part, upon whether two or more levels are used. Early in the study (*1*) it was found that approximately threefold increments in vaccine doses were required to effect significantly different survival rates in successive groups when 10 to 20 mice were used at each level. Such differences in survival were of the order of 15 percent and 50 percent. In practical assays it is desirable to obtain rates which bracket the 50 percent endpoint; ideally, about 15–20 percent through 80–85 percent (*19*). Accordingly, in 2-dose assays a tenfold increment in vaccine dosages is used, while in 3- and 4-dose assays, a threefold increment usually is employed.

The actual quantities of vaccine to be given as immunizing doses are markedly dependent upon the particular test mice available, the dose and virulence of the challenge strain of *S. typhosa*, the virulence enhancing activity of the mucin suspension used, and the environ-

[2] The chemical determinations of nitrogen content were performed by the Department of Biochemistry, Army Medical Service Graduate School, through the courtesy of Lt. Col. M. E. Freeman.

mental conditions pertaining. Quantities required for optimal response vary markedly from laboratory to laboratory and from time to time in the same laboratory. Quantities currently being employed in these laboratories (October 1950) in 2-dose assays are 0.010 ml. and 0.100 ml. at the low and high dosage levels, respectively.

Challenge Suspensions

The challenge organism currently utilized in typhoid vaccine assays is a V form substrain (*14*) of *S. typhosa*, strain 58. Cultures are maintained continuously in the lyophilized state, a separate ampule of dried organisms being used for each assay.

The ampule is opened approximately 21 hours previous to the scheduled time of challenge, and the dried organisms are suspended in a small quantity of tryptose-glucose broth.[3] This seed suspension is inoculated into two 10 ml. tubes of the tryptose-glucose medium and incubated at 37° C. for 4 hours. Following preliminary incubation, 0.2 ml. of one of the actively growing cultures is transferred to each of 5 tubes of tryptose-glucose broth and these, in turn, are incubated at 37° C. for 16 hours.

Approximately 1 hour before the scheduled time of challenge, each of the five broth cultures of *S. typhosa* is mixed thoroughly with a 5 ml. pipette, and 5 ml. from each culture are pooled in a small Erlenmeyer flask. The pooled broth culture is shaken thoroughly, and 1 ml. is transferred to the first of a series of 9 ml. saline dilution blanks. Serial tenfold dilutions then are prepared through 10^7 using a separate sterile 1 ml. pipette for each serial dilution transfer. Nine 1 ml. aliquots of the final 10^7 dilution then are plated with veal infusion agar. These plates are incubated at 37° C. for 24 hours, the colony counts determined, and the average of such counts $\times 10^7$ taken as the most probable viable count per ml. of the original pooled broth culture. Such average counts usually are between 50 and 55 $\times 10^7$ and have been found sufficiently reproducible that, following a few such determinations, the count to be obtained can be predicted with remarkable accuracy.

The final challenge suspension is prepared by adding a calculated quantity (based on the predicted count) of the pooled broth culture, or of one of the lower dilutions, to the desired quantity of sterile 5 percent mucin to yield a final concentration of approximately 1,000 viable *S. typhosa* per 0.5 ml. of the mucin suspension. Care is exercised to use as small an amount of broth culture or saline dilution as is practicable so that dilution of the mucin suspension will be minimal.

The size of the challenge dose (number of viable organisms) has much less effect on mouse survival than does the size of the immunizing

[3] Bacto-tryptose, 2 percent; U. S. P. glucose, 0.5 percent; C. P. NaCl, 0.5 percent; final pH 7.6.

dose. On occasion it has been considered advisable to employ a challenge dose of 10,000 viable *S. typhosa*. Ordinarily the LD_{50} of the challenge organisms for unprotected mice, as determined in these laboratories, is less than 10 organisms.

The inoculated mucin suspension is mixed thoroughly and the viable bacterial content checked by plating dilutions as follows: 1 ml. of inoculated mucin is diluted 1: 100 (two serial tenfold dilutions) with sterile saline and nine 1 ml. aliquots of the final dilution are plated with veal infusion agar. Following incubation for 24 hours at 37° C., the plate counts are determined and averaged. The original viable count of the mucin suspension per 0.5 ml. then is estimated as the average plate count × 50. Any count between 750 and 1,250 is considered a satisfactory approximation of the desired 1,000 challenge dose.

All injections of the challenge suspension are accomplished within 2 hours of the time the 16-hour broth cultures are pooled.

Mucin Suspensions

Mucin suspensions routinely employed in these laboratories for enhancement of virulence of *S. typhosa* are prepared from dehydrated hog gastric mucin (Wilson, Type 1701–W) and are sterilized by intermittent heating. Fifty-gram unit quantities of dried mucin each are homogenized in 1 liter of cold distilled water with the aid of a Waring Blendor. The homogenized material is stored in the refrigerator (4° to 6° C.) for 26 to 30 hours, heated in a bath of boiling water for 5 minutes, filtered through a thin, absorbent cotton-gauze filter to remove gross particles and dispensed in 500 ml. quantities. Following dispensing, the pH is adjusted to 7.3–7.4 with 1N NaOH, and the suspension is sterilized by heating in a bath of boiling water for three 25-minute periods during the next 30 hours. The suspensions are kept in the refrigerator at all times except during actual heating. Tests for sterility, toxicity, and virulence enhancing activity are performed on each batch of mucin suspension prepared.

Experience has indicated that the activity of such suspensions remains essentially unaltered during months of storage under refrigeration. Accordingly, suspensions are prepared in total quantities sufficient (5 to 10 liters) for all assays during an extended period of time.

Test Mice

As far as is known, any strain of mice highly susceptible to experimental infection with virulent strains of *S. typhosa* is satisfactory for use in the potency test. In assays currently being performed in these laboratories, female CFW strain mice (Carworth Farms), weighing 13 to 15 grams, are used routinely. On occasion it is neces-

sary to use male mice, but mixed sexes are not employed in the tests.

The test mice are obtained at least 24 hours prior to beginning the assay. The mice are selected individually at random and are assigned to mouse jars until each jar contains a group of 5 mice. The groups of mice (by jars) are then numbered at random to correspond to numbered spaces on the assay protocol and, furthermore, the jars are assigned at random to locations in the animal test room. The temperature of the room is maintained at 80° F. as closely as practicable.

A minimum of 20 mice (4 groups of 5 each) are employed at each dosage level of each vaccine. An additional 20 mice (4 groups of 5 each) are set aside for use as challenge dose controls. Larger numbers of mice frequently are utilized in critical tests, but an equal number always is assigned to each vaccine-dosage group. A typical assay of a single unknown vaccine thus requires a minimum of 100 mice (20 groups of 5 each) assigned as follows:

Assignment	*Dose*	*Mice*
Unknown vaccine	Low	20
	High	20
Reference vaccine	Low	20
	High	20
Challenge controls		20
Total		100

Immunization and Challenge Procedure

Active immunization of the mice is accomplished by intraperitoneal injection of the vaccine dilutions (in 0.5 ml. volumes). Five ml. syringes graduated to 0.5 ml. and 23 gage, ¾-inch needles have been found convenient for this purpose. The mice selected for challenge dose controls similarly are injected with 0.5 ml. of sterile normal saline.

The mice are challenged 6 days following immunization. The inoculated mucin suspension is administered intraperitoneally by means of a 5 ml. syringe graduated to 0.5 ml. and a 22 gage, 1-inch needle. All mice, including the nonimmunized controls, are injected by groups in random order.

Recording Results

The mice are checked daily for 3 days and the results, in terms of survivors/total in each group at the end of the observation period, are recorded. The procedure and all data pertaining to each assay are recorded in detail in notebooks, but to facilitate maintenance of records and calculation of potency, all essential data and results are summarized on a protocol sheet (see sample form). The data entered on the form are taken from an actual 2-dose assay run on August 24, 1950.

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TYPHOID VACCINE ASSAY PROTOCOL

Assay number	121-11	Vaccine doses:	
Vaccine	Lot 584	Low 0.015 ml.	High 0.150 ml.
Reference	L. S.-1	Mucin lot	305
Mice	CFW, F, 13-15 gm.	Challenge	58, 1000/0.5 ml.
Immunization date	24 Aug., 1950	Actual count	1200/0.5 ml.
Challenge date	30 Aug., 1950	Relative potency	165%±82.5%

Results

(Survivors/Totals)

Lot 584				Reference L.S.-1				Challenge dose controls	
Low dose		High dose		Low dose		High dose			
(1) 2/5	(2) 2/5	(3) 4/5	(4) 5/5	(5) 1/5	(6) 1/5	(7) 4/5	(8) 4/5	(9) 1/5	(10) 0/5
(11) 1/5	(12) 1/5	(13) 2/5	(14) 5/5	(15) 2/5	(16) 0/5	(17) 4/5	(18) 2/5	(19) 0/5	(20) 0/5

Operator.....

Typical protocol of mouse-protection potency assay of typhoid vaccine.

Analysis and Interpretation of Results

1. Acceptability of Data

Assay data, such as are obtained in the tests described here, can be subjected to formal statistical analysis to determine their suitability for calculation of relative potency. However, such analyses are time consuming and, in general, the same safeguards can be accomplished arbitrarily by prescribing certain criteria for acceptability of data. The following criteria have been found entirely practicable and currently are followed in these laboratories.

Survival of Immunized Mice Prior to Challenge

At least 90 percent (18 of 20) of all mice originally assigned to each vaccine-dosage or challenge-dose control group must survive from the beginning of the assay until the time of challenge. Deaths prior to challenge are very rare among mice obtained from healthy stock and given proper care.

Survival of Challenge-Dose Control Mice

Not more than 15 percent (3 of 20) of the nonimmunized control mice shall survive the 3-day period following challenge. Usually 100 percent succumb, but individual mice highly refractory to experimental infection with *S. typhosa* occasionally are observed.

Survival of Immunized Mice Following Challenge

Among mice given the reference vaccine, the results must reveal a definite increase in survival with increased vaccine dose. Furthermore, less than 50 percent of those given the lowest dose of vaccine and more than 50 percent of those given the highest dose must survive the 3-day period following challenge. Thus, in a 2-dose assay where 20 mice are used at each dosage level, the maximum acceptable ranges of survival at the low and high dosage levels are 0 to 9 of 20 (0 to 45 percent) and 11 to 20 of 20 (55 to 100 percent), respectively. Ideally, the survivals at the low- and high-dosage levels should be about 3 of 20 (15 percent) and 17 of 20 (85 percent).

The survival of mice given the unknown vaccine likewise must meet the foregoing criteria if an estimate of relative potency is to be obtained. On occasion, such as when testing high potency experimental vaccines, survival at both (or all) dosage levels may be so great that the endpoint of 50 percent survival is not bracketed, i. e., more than 50 percent of mice given the lower dose(s) of vaccine will survive. When experience indicates that such results truly are due to high potency and are not resulting from sampling variation, preliminary dilutions of the unknown vaccine should be made so as to bring the dosage-response data into general conformity with the foregoing. Since the primary objective of a potency test usually is to determine merely whether or not the unknown equals or exceeds the reference vaccine in potency, results such as are referred to could be considered acceptable for release of the vaccine on test.

2. Analysis of 2-Dose Assay Data—The Knudsen-Curtis Method

Several methods of calculating the relative potency of vaccines are available (*8*, *10*, *13*, *16*). These procedures differ somewhat in regard to details, such as to the particular assumptions underlying the computational procedure and the particular transformation of dosage-mortality data used, but the end results as calculated by the different methods generally are in close agreement. It has been the experience in these laboratories that, provided good data are available, such as those meeting the previously stated criteria for acceptability, it is a matter of little consequence just which computational procedure is employed. In fact, even when just the approximate ED_{50} values of both the unknown and the reference vaccine are estimated by a simple

*Transformation of percentage to angle values**

[The angle values in the body of the table correspond to each percentage of response (survival) listed along the left edge and top. *For example:* the angle value corresponding to 15 percent is 22.8]

Percent	0	1	2	3	4	5	6	7	8	9
0	0	5.7	8.1	10.0	11.5	12.9	14.2	15.3	16.4	17.5
10	18.4	19.4	20.3	21.1	22.0	22.8	23.6	24.4	25.1	25.8
20	26.6	27.3	28.0	28.7	29.3	30.0	30.7	31.3	31.9	32.6
30	33.2	33.8	34.4	35.1	35.7	36.3	36.9	37.5	38.1	38.6
40	39.2	39.8	40.4	41.0	41.6	42.1	42.7	43.3	43.9	44.4
50	45.0	45.6	46.1	46.7	47.3	47.9	48.4	49.0	49.6	50.2
60	50.8	51.4	51.9	52.5	53.1	53.7	54.3	54.9	55.6	56.2
70	56.8	57.4	58.1	58.7	59.3	60.0	60.7	61.3	62.0	62.7
80	63.4	64.2	64.9	65.6	66.4	67.2	68.0	68.9	69.7	70.6
90	71.6	72.5	73.6	74.7	75.8	77.1	78.5	80.0	81.9	84.3
100	90.0									

*With permission, abridged from table 16.8, Snedecor (*25*).

approximate graphical method (*19*), the ratio of the reciprocals of these endpoints generally is in close agreement with the relative potency calculated by more exacting but far more laborious means. Such an approximate method does not provide an estimate of the error of the assay, however, and, as such estimates frequently are useful in interpreting the results of assays on individual lots of vaccine, it is considered advisable to employ one of the more complete computational methods.

The method found most generally useful in these laboratories is that proposed by Knudsen and Curtis (*13*). The Knudsen-Curtis method involves the transformation of percentage response data (survival or mortality) to angle values (*6*), as given in the table, and is adaptable to assays based on several dosage levels but is particularly advantageous for calculating the results of 2-dose assays.

The original publication (*13*) should be referred to for full details, but calculations can be performed readily with no further guidance than that provided here in synopsis form.

Relative Potency

Estimation of relative potency is accomplished by means of the following equation:

$$(1) \qquad \text{Potency as percent of standard} = \text{antilog}\left(2+\frac{iV}{W}\right)$$

where: i=log ratio of doses, i. e., log (high dose/low dose).

$V = U_L - S_L + U_H - S_H$.

$W = S_H - S_L + U_H - U_L$.

and

U_H=response to high dose of the unknown

U_L=response to low dose of the unknown.

S_H=response to high dose of standard.

S_L=response to low dose of standard.

All in terms of angle values.

An example of the use of the Knudsen-Curtis method in calculating the relative potency of an unknown lot of typhoid vaccine from the results of a 2-dose test is afforded by the data presented in the sample form and summarized as follows:

Vaccine	Lot 584		Standard	
Dose (ml.)	0.015	0.150	0.015	0.150
Response:				
Survivors/total	6/20	16/20	4/20	14/20
Percent survival	30	80	20	70
Angle values	33.2	63.4	26.6	56.8

Calculation of relative potency by means of equation (1):

$$i=\log(\text{high dose/low dose})=\log\frac{0.150}{0.015}=\log 10=1.$$

$$V=U_L-S_L+U_H-S_H=33.2-26.6+63.4-56.8=13.2$$

$$W=S_H-S_L+U_H-U_L=56.8-26.6+63.4-33.2=60.4$$

$$\text{Potency as percent of standard}=\text{antilog}\left(2+\frac{iV}{W}\right)$$

$$=\text{antilog}\left(2+\frac{(1)(13.2)}{60.4}\right)$$

$$=\text{antilog}\ (2+0.2185)$$

$$=165\text{ percent}$$

Standard Error of the Assay

Calculation of the standard error of the assay in a 2-dose test requires three values:

1. A constant, 2.303.
2. The standard error of the log ratio of potency, symbolized as σ_M.
3. The relative potency in percent, calculated as above.

The standard error of the assay then is calculated by the following equations:

(2) Standard error of the assay = (2.303) (σ_M) (potency)

The value of σ_M is determined as follows:

$$(3)\qquad (\sigma_M)^2=\frac{(i)^2(57.3)^2}{n}\times\frac{(W^2+V^2)}{W^4}$$

where: i, V and W are the same as previously given.

n = the number of mice at each dose.

$(57.3)^2=3283.3$ = a constant.

A computational example again is afforded by the data presented in the sample form and by the foregoing calculation of relative potency from these data. First, using equation (3):

$$(\sigma_M)^2=\frac{(i)^2(57.3)^2}{n}\times\frac{(W^2+V^2)}{W^4}$$

$$= \frac{(1)^2(57.3)^2}{20} \times \frac{[(60.4)^2 + (13.2)^2]}{(60.4)^4}$$

$$= (164.16) \times (0.000287)$$

$$= 0.0471$$

and

$$\sigma_M = \sqrt{0.0471} = 0.217$$

Then substituting appropriate values in equation (2):

Standard error $= (2.303)\ (\sigma_M)$ (potency in percent)
$= (2.303)\ (0.217)\ (165)$
$= 82.5$ percent

Thus, the potency of vaccine lot 584 in relation to the reference standard is 165 percent ± 82.5 percent.

Notes

A. In all 2-dose assays performed identically as here described (20 mice per group and a tenfold increment between low and high dose) the equation for calculation of $(\sigma_M)^2$ can be simplified further as all items in the expression $\frac{(i)^2(57.3)^2}{n}$ are constant. Thus the equation reduces to

$$(\sigma_M)^2 = 164.16 \times \frac{(W^2 + V^2)}{W^4}$$

Substituting the appropriate values from the foregoing example:

$$(\sigma_M)^2 = 164.16 \times \frac{[(60.4)^2 + (13.2)^2]}{(60.4)^4}$$

$$= 0.0471$$

the same value as previously obtained.

B. When 1 or 2 mice of a vaccine-dosage group die prior to challenge, angle values corresponding to the actual percentage of survivors out of the total tested are employed. However, when calculating the standard error of the assay, the slight discrepancy in the value of n which results from the unequal numbers in the various groups is ignored without introducing appreciable error.

3. Interpretation of Results

Acceptable Potency

Following completion of the inspection and analysis of the assay data, the unknown vaccine is considered of acceptable potency provided:

A. The assay data are acceptable in accordance with the prescribed criteria.

B. The relative potency of the unknown is at least 60 percent of that of the reference vaccine.

Potencies ranging from 60 to 75 percent of the reference standard are considered borderline. In these cases, the calculated percentage relative potency must not differ from the expected potency of 100 percent by more than twice the standard error of the assay.[4]

Repeat Tests

In case an assay is unsatisfactory because of unsuitability of data, the results are ignored and the test is repeated with such adjustment of vaccine dosage as may be indicated.

When the assay results meet the requirements for suitability of data but the relative potency is less than 60 percent of the reference vaccine, or the potency is borderline (60 to 75 percent) and the difference between the calculated potency and the expected value of 100 percent is more than twice the standard error of the assay, the test is repeated employing twice as many mice (at least 40) at each vaccine-dosage level of both the unknown and reference vaccine. Such repeat tests are performed as two separate assays and the relative potency is calculated independently in each case. For acceptability, the results of both assays must meet requirements A and B as specified above.

Assays in which the data are suitable but the potency of the unknown is less than 60 percent of the reference standard are not repeated more than once.

Discussion

The assay procedure presented for determining the immunogenic potency of typhoid vaccines has been adopted tentatively by the Biologic Products Division, Army Medical Service Graduate School, since it is considered to be a practical compromise procedure which will yield a maximum of information consistent with reasonably economical laboratory management. Extensive experience with this procedure has revealed that the results obtained are more closely reproducible than those obtained with procedures previously employed. Furthermore, it has been found possible, by deliberate experimentation, to distinguish between vaccines of known different immunogenic activity for the mouse.

The basic requirements of a satisfactory biological assay have been presented in outline form by Bliss (7). The following is the essence

[4] This limit was established more or less arbitrarily for the sake of convenience and corresponds to approximately the 2.5 percent significance level. If it is desired to use the 5-percent significance level for rejection, the determined relative potency must not be less than 100 percent by more than 1.645 times the standard error of the assay.

of these requirements as reviewed by Emmens (*9*) with general comments as to the degree the reported procedure meets or fails to meet these requirements:

1. "Different samples of the same drug should show the same relative potencies in biological assay as under clinical test." The proposed method in no way meets this requirement, a criticism equally applicable to all laboratory assays of this type.

2. "On the co-ordinates used for biological assay the curve relating response to log dose should be a straight line and relatively steep when compared with the variation about the line." The proposed procedure, being a 2-dose test, does not provide for any test for linearity of the dosage-response curve. However, extensive preliminary studies (*1*, *2*) have established with adequate certainty that in this type of assay the relationship between log dose and survival in terms either of probits or angle values is linear. The steepness (slope) of the line is a function of the heterogeneity of the mice employed and cannot be changed by mere redesign of the assay procedure.

3. "The potency of the unknown or sample should be determined by comparative tests with a stable reference standard and expressed in units of this standard." This requirement is met fully and, in the authors' opinion, constitutes the most important feature of the method.

4. "The living material exposed to different doses of the standard and unknown must be as nearly equivalent as it can be made. Potential sources of variation, such as differences between individuals, litters, dates of treatment and sexes, should never coincide or be confounded with differences in treatment, but within these limitations the dosages and samples must be assigned at random." The specific materials and randomization procedures employed were so selected as to meet this requirement.

5. "A determination of potency should always include an estimate of its error, computed as an integral part of the assay." This requirement likewise is met and agreed upon in principle. However, it is questioned whether or not such an estimate truly serves the purpose the uninitiated reader logically may assume it does. The error of the assay, as calculated, is essentially a theoretical value based upon the expected vagaries of sampling from a binomially distributed population and does not take into account between-tests and other types of variation which are the bane of the assayist's existence. Stating it mildly, it is disquieting to the laboratory worker to obtain results from two tests on the same product which differ markedly more than would be expected from the estimated error of either.

To the foregoing, Emmens (*9*) adds an additional requirement ". . . that the assay methods should give reproducible results, which

should be consistent with internal indications of precision, and that this precision should not alter violently from test to test or from time to time." The merit of this requirement is granted and, if "reproducible" is considered a relative term, the requirement is attainable by the proposed method. Reproducibility can be increased, of course, by employing 3, 4, or even more vaccine-dosage levels and large numbers of mice at each level. However, the cost of such assays severely limit their practical value. Likewise, determination of the consistency of internal indications of precision with the reproducibility of results, while certainly a laudable objective, requires extensive statistical analysis which not only is time consuming but also is beyond the present level of training and experience of the overwhelming majority of individuals charged with the responsibility for conducting such assays. Employment of certain criteria for acceptability of assay data, as prescribed, does much to alleviate the necessity for extensive statistical analysis even though such restrictions do introduce an element of bias.

It has been found in these laboratories that differences in the susceptibility of mouse strains, virulence of challenge organisms, virulence enhancing activity of different mucin suspensions, and other experimental factors exert a profound effect on absolute estimates of potency such as ED_{50} values. Further experiments, to be reported elsewhere, have indicated that, in general, such effects can be eliminated or controlled by use of a reference vaccine and by expressing the potency of an unknown in relative terms in respect to the standard. It is not known, however, how such factors affect the precision of quantitative differentiation between products truly differing in potency. Studies in this regard are now in progress.

In the choice of a computational procedure, full consideration was given to the level of mathematical understanding and skill required and to the time and effort involved in calculations. The Knudsen-Curtis method was selected as the procedure of choice since experience has indicated that results of analyses by this method were in close agreement with those obtained by other statistically valid methods and the computational procedures were less tedious, particularly when dealing with the results of a 2-dose assay. While it is advantageous to employ an electric calculator in such analyses, no computations are involved in analysis of 2-dose assay data that are not readily performed with logarithms.

The true nature of an "error of the assay" is not clearly understood by many laboratory workers. The standard error is a measure of the degree of variability that could be expected in repeat tests performed under identical conditions. However, even under identical conditions, only two-thirds of such repeat tests theoretically should fall between the limits: (a) expected percent potency minus the standard error and (b) expected percent potency plus the standard error. Ninety-five

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percent of repeat tests should fall between the broader limits: (a′) expected percent potency minus twice the standard error and (b′) expected percent potency plus twice the standard error. Unfortunately, identical conditions seldom (if ever) obtain, and in repeat tests actually performed in the laboratory even a greater range of values not uncommonly is experienced.

In a study recently completed in these laboratories (*5*), a series of six pairs of identical assays was performed on a reference vaccine, each pair of assays being conducted at a different time. The members of each pair were designated as A or B prior to performing the tests and, from the resultant data, the relative potency, B as percent of A, was calculated in each case. The expected relative potency was 100 percent in each instance, of course. The relative potencies, as calculated, ranged from 51 percent to 195 percent; a range in reasonable agreement with what would be expected from the standard errors of individual tests.

The lower limit of acceptable potency, 60 percent of that of the reference standard, was not established in a purely arbitrary manner. This level was approximated from the calculated standard deviation of the six relative potency values determined on the reference vaccine just referred to, i. e., the expected relative potency of 100 percent minus one standard deviation, as calculated from the series of relative potencies, approximated 60 percent. Consequently, it is to be expected that if assays were run on a series of vaccines all of which were exactly equal in potency to that of the reference standard, approximately 16 percent would fail to pass the test as a result of testing variation alone. However, it is quite improbable (about 1 chance in 40) that any single vaccine would fail to pass both an original and a repeat test if the potency actually were equal to that of the reference standard.

Summary

The assay procedure for the routine determination of potency of typhoid vaccine tentatively adopted by the Biologic Products Division, Army Medical Service Graduate School, is presented in detail.

The assay is a 2-dose active-immunity mouse-protection test in which the potency of an unknown is determined in direct comparison with that of an arbitrarily selected reference standard vaccine.

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A Rapid Flocculation Test for the Diagnosis of Trichinosis

By John Bozicevich, M. A., John E. Tobie, Ph. D., Elizabeth H. Thomas, B. A., Helen M. Hoyem, B. A., and Stanley B. Ward*

The value of complement fixation and precipitin reactions as aids in the diagnosis of trichinosis has been pointed out by Bozicevich (*1*), Culbertson (*2*), and others. However, certain of these tests have disadvantages, including the long period of time before results are obtained, the occurrence of nonspecific reactions, and the requirement of highly trained personnel. Suessenguth and Kline (*3*) described a flocculation test for trichinosis in which the antigen consisted of cholesterin crystals coated with an alkaline aqueous extract of powdered trichina larvae. In our hands, their flocculation test gave some falsely positive reactions, and the antigen was not stable. It is hoped that the flocculation test herein described may overcome many of these difficulties so that a rapid and specific test for the diagnosis of trichinosis will be available.

Materials and Methods

Preparation of Trichina Extract

1. Infect medium-sized adult rats by inoculating intragastrically with infective larvae of *Trichinella spiralis*.
2. At the end of 2 months sacrifice animals; skin and eviscerate.
3. Examine muscle samples for larvae, and use only heavily infected carcasses.
4. Put carcasses through meat grinder.
5. Add 70 gm. of ground meat to a battery jar containing 3 liters of artificial gastric juice. The digestive fluid is prepared by adding 21 ml. of HCl (specific gravity 1.10–1.19) to 3 liters of tap water (37°–40° C.) and then adding 15 gm. of pepsin.
6. Slowly agitate the mixture, by means of a stirring apparatus, for 6 hours at 37° C.
7. Pour mixture through one layer of surgical gauze (35 to 40 mesh apertures to the inch) into a 3-liter glass funnel, the stem of which is fitted with a 15-ml. conical centrifuge tube by means of a rubber tube.

*From the Laboratory of Tropical Diseases, Microbiological Institute of the National Institutes of Health, Public Health Service. Valuable assistance was provided by J. Robert Buchheit in the performance of the flocculation test.

8. Allow mixture to stand for 1½ hours so that the larvae will settle to the bottom of the centrifuge tube.

9. Close a pinchcock on the rubber tube between the funnel stem and centrifuge tube and remove the tube containing the larvae.

10. Pipette off supernatant fluid as closely as possible to the settled larvae; refill tube with physiological saline, and allow to settle for 10–15 minutes. Repeat this procedure (usually 5 to 6 times) until the larvae are free of rat protein.

11. Suspend 2 ml. of settled larvae in 8 ml. of physiological saline and transfer to a 10-ml. Ten Broeck tissue grinder.

12. Thoroughly grind larvae; transfer to flask containing 90 ml. physiological saline, and allow to extract at 4° C. for 24 hours.

13. Centrifuge the suspension in 20-ml. culture tubes at approximately 3,000 rpm for 15 minutes (International Centrifuge, size 2, No. 240 head).

14. Pour off and save supernatant; discard sediment.

15. Centrifuge the supernatant at 15,000 rpm for 15 minutes in an International Multispeed Centrifuge or Servall Centrifuge, Model SS–2. If a high-speed centrifuge is not available, the suspension can be filtered through a sintered glass filter of fine porosity (UF). Filters which readily adsorb protein are not satisfactory. The supernatant fluid or the filtrate, as the case may be, is the trichina extract.

Preparation of Stock Bentonite

1. Suspend 0.5 gm. of BC micron or No. 200 standard Volclay (Wyoming bentonite) [1] in 100 ml. of distilled water.

2. Homogenize in Waring Blendor for 1 minute.

3. Transfer bentonite suspension to 500-ml. glass-stoppered graduate and add distilled water to make 500 ml.

4. Shake thoroughly and allow to settle for 1 hour.

5. Pour off and save supernatant, discard sediment.

6. Pour supernatant into 100-ml. centrifuge tubes and centrifuge, by tachometer, at 1,300 rpm for 15 minutes. Use International Centrifuge, size 2, with No. 240 head.

7. Pour off and save supernatant; discard sediment.

8. Centrifuge supernatant in 100-ml. tubes at 1,600 rpm for 15 minutes.

9. Pour off supernatant and discard it.

10. Resuspend sediment in 100 ml. distilled water and homogenize in Waring Blendor for 1 minute. This is the stock bentonite which,

[1] We wish to express our appreciation to Paul Bechtner of the American Colloid Co., Merchandise Mart Plaza, Chicago 54, Ill., for generously supplying samples of Volclay and information concerning the properties of the product.

in our experience, has remained stable for as long as 4 months without losing its adsorptive properties.

When aliquot samples from the same lot of bentonite were suspended in identical volumes of water, different densities were obtained. Attempts to standardize bentonite suspensions by spectrophotometric methods were unsuccessful. It is important to have colloidal particles of the size which are obtained by centrifugation at 1,300–1,600 rpm (relative centrifugal force of approximately 500 to 750). The larger colloidal particles obtained by centrifugation at speeds of less than 1,300 rpm tend to flocculate spontaneously, thus giving falsely positive reactions in the test, while the smaller particles, obtained by centrifugation at speeds greater than 1,600 rpm, do not tend to flocculate even in the presence of a positive serum, thus giving falsely negative reactions. Furthermore, the smaller particles often float, making it difficult to read the test. We have noted that if the stock bentonite, which has been suspended in 100 ml. of fresh distilled water after the 1,600 rpm centrifugation, is now centrifuged at 1,300 rpm, practically all of the particles are thrown down. Apparently changes occur in the physical properties of the bentonite; however, these changes do not interfere with the performance of the test. It should be stressed that distilled water only should be employed in the bentonite preparation; otherwise, if electrolytes are used in the process, the bentonite may flocculate spontaneously.

Preparation of Stock Antigen

1. Add 10 ml. of trichina extract to 20 ml. of stock bentonite and allow to adsorb at 4° C. for 15 to 18 hours.

2. Add 5 ml. of a 0.1 percent Thionin Blue O (C. I. No. 926) solution, prepared in distilled water, to the above 30 ml. of sensitized bentonite. A variety of basic and acidic dyes were tried but only certain of the basic dyes proved satisfactory.

3. Shake thoroughly and allow 1 hour for adsorption of the dye. This is the stock antigen. In our experience, this antigen has retained its antigenic properties for relatively long periods of time if kept at 4° C., but it has a tendency to flocculate spontaneously.

Preparation of Slides and Antigen Pipettes

By means of a multiple ring maker prepare twelve wax rings on a clean 3- by 2-glass slide. The wax is of the composition recommended by Mazzini (*4*), 50-gm. sealing wax to 20-gm. paraffin. Before preparation of the wax rings the slides should be meticulously cleaned with a mild abrasive cleanser and rinsed in alcohol to free them of grease.

The pipettes used in dispensing the antigen are prepared from pyrex glass tubing with 3- to 4-mm. inside diameter. Each pipette is

pulled out to a diameter which will permit a delivery of 60 to 80 drops of antigen per ml.

Preparation of Test Antigen

1. Thoroughly shake stock antigen and transfer 8-ml. to a 16- by 150-mm. culture tube.

2. Add 10 ml. of physiological saline, shake thoroughly and centrifuge for 5 minutes at 2,500 rpm (International Centrifuge, size 2, No. 240 head).

3. Discard supernatant fluid and resuspend sediment in 15 ml. of physiological saline.

4. Centrifuge for 5 minutes at 2,500 rpm as before and discard supernatant.

5. Resuspend sediment in 4 ml. of physiological saline.

6. *Antigen adjustment.* Up to this point in its preparation, the antigen has a tendency to flocculate spontaneously. In order to keep the colloidal particles dispersed, it is necessary to add a nonionic surface active agent. In our experience, the anionic and cationic agents were unsatisfactory. Add 0.5 ml. of a 1 percent distilled water solution of Tween 80 (polyoxyethylene sorbitan monooleate) to the 4 ml. of antigen suspension and shake thoroughly.

A saline and negative serum control must be performed to determine whether or not the Tween 80 has adjusted the spontaneous flocculating properties of the antigen.

Saline control. Add 1 drop of antigen, using the antigen pipette, to 0.1 ml. of physiological saline on a wax-ringed slide, place on a rotating apparatus (Boerner type) and rotate 100 to 120 times per minute for 15 minutes. If a reaction greater than 2+ is obtained, additional 0.1 ml. amounts of Tween 80 must be added, not to exceed 0.5 ml. or 1.0 ml. in total, until a 2+ or less reading is obtained. In case an antigen does not adjust to these requirements, it should be washed, by centrifugation, several times in distilled water, 4 ml. physiological saline and 0.5 ml. Tween 80 added as before, and again tested against saline for its flocculating properties.

Negative serum control. Add 1 drop of antigen, using the antigen pipette, to each 0.1 ml. amount of twofold serial dilutions of a negative serum. The serial dilutions are made in physiological saline and should be the same as those employed for the positive serum in "the test proper." Place on rotating apparatus as before and read at end of 15 minutes. There must be no flocculation in the dilution of the negative control serum corresponding to the highest dilution of the positive serum which gives a 3+ or greater reaction. If an antigen is prepared as outlined and

meets the above requirements, it is ready for use as the test antigen. In the present study the test antigens retained their antigenic properties for as long as 2 months but the stock antigens for a much longer period of time when both were kept at 4° C. It therefore seems better to prepare small amounts of test antigen and to retain the bulk of the antigen as stock antigen.

7. Test the antigen against twofold serial dilutions of a positive serum according to the technique outlined in "the test proper."

The Test Proper

1. Prepare 1:5, 1:10, and 1:20 serum dilutions with physiological saline. If desired the twofold serial dilutions may be continued to the endpoint.

2. Pipette 0.1 ml. of each dilution onto a wax-ringed slide by putting the pipette in contact with the slide, delivering the 0.1 ml. amount, and withdrawing. The same pipette is used for all dilutions of the same serum by proceeding from the highest to the lowest.

3. The test antigen, which is kept at 4° C., should be removed from the refrigerator and allowed to reach room temperature before use.

4. With an antigen pipette add one drop of the test antigen to the center of each wax ring containing the diluted serum. Before the antigen is dispensed it should be shaken vigorously to obtain a homogeneous suspension. Each time the antigen is used the shaking procedure should be repeated.

5. Place the slide on a rotating apparatus (Boerner type) and rotate 100 to 120 times per minute for 15 minutes. If necessary the slides can be rotated by hand, but slightly lower titers may be obtained.

6. The flocculation reactions are then read.

7. For each series of tests two controls are used:

(a) *Negative serum control.* By means of an antigen pipette, add 1 drop of antigen to 0.1 ml. of each of the same serum dilutions as used for the test sera.

(b) *Saline control.* By means of an antigen pipette add 1 drop of antigen to 0.1 ml. of physiological saline used in the dilution of the sera.

Low-power magnification of the compound microscope is used in reading the reactions. Because of the effect of rotation, the flocs in a positive serum may be concentrated at one side of the wax ring and because of this, it is essential that the entire area of the ring be examined. In a 4+ reaction all of the sensitized particles will be flocculated into separate masses. The flocs themselves may vary in size depending upon the titer of the serum, but, in any case, the fields between the flocs are clear. Approximately three-fourths of the sensitized particles are flocculated in a 3+ reaction, one-half in a 2+ reaction, one-fourth in a 1+ reaction, and none in a negative reaction.

The dispersion of colored particles in a negative serum gives a slight blue haze, but the concentration of the colored particles of the floc in a positive serum gives a very intense blue color which is discernible immediately through the microscope and often macroscopically as well. A serum which gives a 4+ or 3+ reaction is considered positive while a serum which gives a 2+ or 1+ reaction is considered negative.

Results and Discussion

In order that we might be able to determine the specificity and sensitivity of the flocculation reaction, it was necessary to compare it with some other serological reaction. For some years this laboratory has utilized principally the complement fixation test for the diagnosis of trichinosis because it has been found to give more reliable results than the precipitin test. For the evaluation of the flocculation test, the complement fixation test was, therefore, used as our standard for comparison.

A few of the sera used in the evaluation of the flocculation test for trichinosis were fresh, but the majority had been stored for from 1 to 5 years. The stored sera had been merthiolated and maintained at temperatures of 4° C. during the storage period. Most of the sera were tested when fresh by the complement fixation reaction, and then retested for the present study after the period of storage. The sera used as positive test sera were either from patients with a clinical diagnosis of trichinosis or suspected of having trichinosis. Several of the diagnoses had been confirmed by demonstration of the larvae of *T. spiralis* at biopsy. The negative control sera were from patients found negative by complement fixation for trichinosis and/or amoebiasis.

Part a of the table compares the results of the flocculation and complement fixation reactions obtained on 127 of the sera which were tested after a long period of storage. Twenty-two of the sera were positive by both the flocculation and complement fixation tests, and 74 were negative by both tests. However, 13 were negative by the flocculation test but positive by the complement fixation test, and conversely 18 were positive by flocculation but negative by complement fixation. It should be stated that of these 18 sera 15 were positive by the complement fixation test when the fresh sera were originally tested. This would seem to indicate that these were true positive flocculation reactions. The remaining 3 sera were not tested by complement fixation when the sera were fresh, but only after storage. Since the flocculation tests were performed only on stored sera, results of these tests on fresh sera are not available to explain the 13 sera negative by flocculation but positive by complement fixation. Statistical analysis of the data showed that if it is assumed that the complement fixation test detects antibodies produced in trichi-

nosis, then it can be assumed that the flocculation test also detects these antibodies.

Part b of the table gives a further analysis of the data by comparing the results of flocculation reactions obtained on 109 of the sera, which were tested after a long period of storage, to results of complement fixation reactions on the same sera when fresh. Of 70 sera originally positive by the complement fixation test, 37 were found positive by the flocculation test performed on the sera after a long period of storage. The flocculation test had detected more than 50 percent of the positives. Analysis of the data presented in part c of the table shows that the complement fixation test itself, when similarly compared, was able to detect somewhat less than 50 percent of the positives, 32 of an original 67. Thus, we should not expect the flocculation test to do much better. The discrepancy between the total numbers of original positive sera, 70 and 67, is explained by the fact that 3 of the stored sera, when retested by complement fixation, gave anticomplementary reactions with the result that the data could not be included. The above comparisons show that the flocculation and complement fixation tests compare favorably and that if the complement fixation test detects infection with *T. spiralis*, so does the flocculation test. From part b of the table it is noted that the flocculation reaction on stored sera was never positive when tested against fresh sera that were negative by complement fixation; thus, the flocculation test apparently gave no false positives. Perusal of the data in part c

Comparison of results of flocculation and complement fixation tests performed on 139 sera [1]

PART a. Flocculation and complement fixation reactions obtained on 127 of the sera tested after a long period of storage:

		Complement fixation (stored sera)	
		Positive	*Negative*
Flocculation (stored sera)	*Positive*	22	18
	Negative	13	74

PART b. Flocculation reactions obtained on 109 of the sera, tested after a long period of storage, compared with results of complement fixation reactions on the same sera when fresh:

		Complement fixation (fresh sera)	
		Positive	*Negative*
Flocculation (stored sera)	*Positive*	37	0
	Negative	33	39

PART c. Complement fixation reactions obtained on 97 of the sera tested when fresh and after a long period of storage:

		Complement fixation (fresh sera)	
		Positive	*Negative*
Complement fixation (stored sera)	*Positive*	32	0
	Negative	35	30

[1] Because certain tests were not performed and because of anticomplementary reactions in the complement fixation test, the reactions recorded in each of the three parts of the table do not total 139.

similarly shows that complement fixation tests performed on stored sera gave no falsely positive reactions—the tests negative on fresh sera were still negative on the same stored sera.

For purposes of determining the titer, each serum was prepared in twofold serial dilutions to the endpoint. The titers varied from serum dilutions of 1:5 to 1:2560. Approximately half of the positive sera had titers of from 1:5 to 1:40, while the remaining half exhibited titers of 1:80 to 1:2560. Of all the positive sera tested by the flocculation reaction, only two sera of high titer gave zonal reactions. In such cases it is desirous to set up quantitative titrations by continuing the twofold serial dilutions to the endpoint. Usually a weak flocculation reaction will be evident in the lower dilutions of a serum exhibiting zonal tendencies. Merthiolate and phenol, in the amounts used as serum preservatives, have not interfered with the flocculation reaction. Grossly particulate serum may interfere with the reaction; hence, it is advisable to centrifuge each serum immediately before use. The anticomplementary tendencies of chylous serum makes such serum unsatisfactory in the complement fixation reaction. It has been our experience that chylous serum can be used in the flocculation test since it does not interfere with this reaction. Both inactivated and uninactivated serum were found satisfactory.

The present data do not indicate a superiority of the flocculation test over the complement fixation test, but if they are equally as sensitive and specific, the outstanding advantages of the former should make it the test of choice. With the flocculation test, results can be obtained in a matter of minutes and its performance does not require highly trained personnel. It seems likely that the antigen can be prepared commercially, and, if so, it will be possible for almost any laboratory, regardless of size, to perform the test. It is our impression that the flocculation test may prove more sensitive than the complement fixation test. However, a large series of tests on fresh sera, using both techniques, must be performed to prove or disprove this impression.

In regard to the use of the flocculation test for other diseases, preliminary studies indicate that it may be applied to the diagnosis of brucellosis, tularemia, and certain parasitic diseases besides trichinosis.

Summary and Conclusions

1. A rapid flocculation test for the diagnosis of trichinosis is described in detail.

2. Comparison of the flocculation test with the complement fixation test indicated that they were equally sensitive and apparently gave no falsely positive reactions.

3. The flocculation test has several outstanding advantages in-

cluding the rapidity with which it can be performed and the fact that highly trained personnel are not required.

4. In our hands, the antigen has remained stable for months and if it can be prepared commercially, the flocculation test for trichinosis should be a valuable diagnostic aid to both the large and small laboratory.

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Incidence of Disease

No health department, State or local, can effectively prevent or control disease without knowledge of when, where, and under what conditions cases are occurring

UNITED STATES

Reports From States for Week Ended June 2, 1951

For the current week 116 cases of poliomyelitis were reported as compared with 133 for the same week in 1950, and 156 in 1949. States reporting 5 or more cases for the current week were: California, 25; Texas, 22; Florida, 9; Georgia, 8; and Alabama, 5. Four of these are southern States in which the seasonal upswing would be expected to appear earlier than in northern areas.

Rocky Mountain spotted fever cases were fewer for the current week, 15, compared with the same week last year when 23 were reported.

Twelve cases of infectious hepatitis were reported, six in New York State, and two each in Florida, Ohio, and Utah. Wyoming reported one case of Colorado tick fever.

Epidemiological Reports

Streptococcal Sore Throat

Dr. Donovan, District Health Officer, Geneva, N. Y., has reported an outbreak of streptococcal sore throat in a Veterans Hospital in the central part of the State. About 150 cases occurred in a 3-day period, May 26 to 28. The illnesses were mild and all cases have recovered. The suspected source of infection was a food handler who had a sore throat just before the outbreak occurred. Another food handler had a positive throat culture.

Rabies

Dr. A. S. McCown, Virginia Department of Health, reports a very marked decline in the incidence of animal rabies in three adjoining counties, Pittsylvania, Halifax, and Mecklenburg, located in the southern part of the State. The decline took place subsequent to antirabies vaccination and suppression of stray dogs. In 1947, there were 78 cases of rabies in animals in these three counties which were confirmed by laboratory examination. In 1948, there were 70 rabid animals. Rabies control activities, including vaccination, were instituted in September 1948 in one county and 2 months later

in the other two. In 1949, only 3 cases of animal rabies were reported in the three counties; 2 were reported in 1950, and none from January 1 to May 26, 1951.

The first case of human rabies to be discovered in Up-State New York since 1945 occurred recently in a 15-year-old boy residing in the vicinity of Coxsackie. The diagnosis was not definitely suspected until autopsy. A family dog is suspected of being the source of infection. The boy lived in an area where animal rabies incidence is relatively high, especially among foxes.

Gastroenteritis

Dr. Burke Diefendorf, District Health Officer, Glen Falls, N. Y., has reported an outbreak of *Staphylococcus aureus* enterotoxin food poisoning affecting school children in northeastern New York. Of 168 pupils and 7 teachers eating in the cafeteria lunch room, 59 pupils and 2 teachers became ill May 18, 3½ hours afterward. There was sudden onset with nausea, with vomiting in some, and mild diarrhea in a few. Potato salad was the suspected vehicle since all of those who became ill ate it. A total of 146 persons ate the salad. It had been prepared at 9 a. m. and remained at room temperature until noon. The laboratory examination of a left-over sample revealed the presence of *S. aureus, Streptococcus fecalis, B. coli,* and certain other organisms.

Dr. J. C. Hart, Connecticut Department of Health, reports two outbreaks of gastroenteritis occurring in two groups of persons who attended banquets on the same day in the same restaurant. They were served the same menu. In one group of 97 persons, 80 became ill, and in the other group of 22, there were 14 who were affected. Diarrhea, abdominal pain, and vomiting occurred after an incubation period of 8 to 16 hours. The suspected vehicle of infection was roast chicken with gravy.

Intestinal Parasites

Dr. J. C. Hart reports that routine stool examinations have been made by the State Bureau of Laboratories on Jamaicans employed as seasonal laborers on tobacco plantations of the State. Four such persons have carried cysts of *Endamoeba histolytica* and 10 had ova of *Necator americanus.* These were found over a period of 3 months.

Influenza

Dr. J. A. Pons, Puerto Rico Commissioner of Health, has reported a gradually increasing incidence of influenza during the past 2 months, which is now assuming epidemic proportions on the island. A total of 7,098 cases was reported for the week ended May 26 compared with 2,090 and 491 for the previous 2 weeks, respectively. The disease has been mild with a low mortality. Specimens have been obtained for isolation of virus.

Serum Hepatitis

It has been reported that 14 cases of serum hepatitis occurred in Portland, Maine, during the past 10 months in persons who had received thrombin of human origin. The illness was found in Boston in another group of persons who had been treated with the same product. The incubation periods in the Portland group varied from 90 to 105 days. Thrombin of human origin has been recalled from all distributing centers and consignees.

Infectious Hepatitis

Dr. W. L. Halverson, California Director of Health, has reported four cases with one death of infectious hepatitis in an institution located in Los Angeles County. The disease occurred to a dentist, an attendant, and two patients. Investigation revealed that the dentist had the infection several months ago. The onset of disease in the attendant was March 1, and in one patient on March 2. The diagnosis was made post mortem, following an examination on March 22 in the other patient.

Comparative Data for Cases of Specified Reportable Diseases: United States

[Numbers after diseases are International List numbers, 1948 revision]

Disease	Total for week ended—		5-year median 1946–50	Seasonal low week	Cumulative total since seasonal low week		5-year median 1945–46 through 1949–50	Cumulative total for calendar year—		5-year median 1946–50
	June 2, 1951	June 3, 1950			1950–51	1949–50		1951	1950	
Anthrax (062)				[1]	[1]	[1]	[1]	36	18	23
Diphtheria (055)	62	75	158	27th	4,674	7,088	10,432	1,767	2,817	4,074
Encephalitis, acute infectious (082)	20	9	9	[1]	[1]	[1]	[1]	370	283	194
Influenza (480–483)	529	946	495	30th	127,748	146,984	146,984	113,206	136,400	126,343
Measles (085)	18,894	13,061	17,967	35th	411,968	239,426	478,392	383,267	220,297	443,446
Meningitis, meningococcal (057.0)	79	72	58	37th	3,153	2,898	2,854	2,192	1,984	1,882
Pneumonia (490–493)	1,122	1,163	[2]	[1]	[1]	[1]	[1]	[3] 39,246	50,732	[2]
Poliomyelitis, acute (080)	116	133	144	11th	[4] 762	880	860	[4] 1,974	2,011	1,226
Rocky Mountain spotted fever (104)	15	23	23	[1]	[1]	[1]	[1]	62	76	88
Scarlet fever (050) [5]	1,379	1,015	1,513	32d	62,748	51,963	75,549	47,057	35,524	53,005
Smallpox (084)		1	1	35th	13	42	66	5	22	45
Tularemia (059)	9	13	25	[1]	[1]	[1]	[1]	[6] 299	434	434
Typhoid and paratyphoid fever (040, 041) [7]	46	84	61	11th	[8] 463	604	604	[8] 898	1,114	1,114
Whooping cough (056)	1,457	2,484	2,079	39th	55,340	79,886	76,130	33,738	58,350	44,864

[1] Not computed. [2] Data not available. [3] Additions for week ended May 26—Pennsylvania, 30 cases; West Virginia, 7; Georgia, 14; week ended May 19, Tennessee, 27. [4] Deductions: Georgia, 1 case each for weeks ended May 12 and May 26. [5] Including cases reported as streptococcal sore throat. [6] Deduction: Arkansas, week ended May 5, 1 case. [7] Including cases reported as salmonellosis. [8] Deduction: North Carolina, week ended May 19, 1 case.

Reported Cases of Selected Communicable Diseases: United States, Week Ended June 2, 1951

[Numbers under diseases are International List numbers, 1948 revision]

Area	Diphtheria (055)	Encephalitis, infectious (082)	Influenza (480–483)	Measles (085)	Meningitis, meningococcal (057.0)	Pneumonia (490–493)	Poliomyelitis (080)
United States	**62**	**20**	**529**	**18,894**	**79**	**1,122**	**116**
New England	**5**	**1**	**3**	**987**	**5**	**14**	
Maine	1		1	35	2	4	
New Hampshire				36			
Vermont				101			
Massachusetts	3	1		543			
Rhode Island				23			
Connecticut	1		2	249	3	10	
Middle Atlantic	**11**	**7**	**7**	**3,919**	**10**	**130**	**7**
New York	4	5	[1] 4	1,820	8	33	4
New Jersey		1	3	945		48	2
Pennsylvania	7	1		1,154	2	49	1
East North Central	**7**	**6**	**4**	**3,884**	**11**	**88**	**11**
Ohio	1			650	4		
Indiana	5		4	82		14	1
Illinois		1		670	3	45	3
Michigan	1	3		502	1	29	3
Wisconsin		2		1,980	3		4
West North Central	**4**		**14**	**1,116**	**4**	**88**	**4**
Minnesota	2			112		6	
Iowa				221			1
Missouri			2	225	1	3	
North Dakota	1		12	164	1	71	
South Dakota				12			
Nebraska				5			3
Kansas	1			377	2	8	
South Atlantic	**13**		**222**	**1,574**	**11**	**360**	**24**
Delaware				37			
Maryland			1	267		20	
District of Columbia				51		3	
Virginia			121	639	2	25	1
West Virginia	1			290	6		
North Carolina	6			39	2		4
South Carolina	3		9	35		3	2
Georgia	3		91	127	1	309	8
Florida				89			9
East South Central	**8**		**18**	**580**	**12**	**74**	**6**
Kentucky	1		3	216	5	20	1
Tennessee	1		13	123	1		
Alabama	5			220	3	34	5
Mississippi	1		2	21	3	20	
West South Central	**7**	**1**	**133**	**2,238**	**19**	**278**	**30**
Arkansas			76	201	3	36	2
Louisiana	1			73	1	25	4
Oklahoma			57	444		24	2
Texas	6	1		1,520	15	193	22
Mountain	**3**	**1**	**86**	**815**		**36**	**6**
Montana			13	96			
Idaho				116			
Wyoming				39		2	
Colorado			3	159		16	3
New Mexico	2			86		5	
Arizona	1	1	70	231		13	3
Utah				84			
Nevada				4			
Pacific	**4**	**4**	**42**	**3,781**	**7**	**54**	**28**
Washington			18	569	1	2	2
Oregon	1		13	536	1	27	1
California	3	4	11	2,676	5	25	25
Alaska			16				
Hawaii				20			

[1] New York City only.

Reported Cases of Selected Communicable Diseases: United States, Week Ended June 2, 1951—Continued

[Numbers under diseases are International List numbers, 1948 revision]

Area	Rocky Mountain spotted fever (104)	Scarlet fever (050)	Smallpox (084)	Tularemia (059)	Typhoid and paratyphoid fever [1] (040, 041)	Whooping cough (056)	Rabies in animals
United States	**15**	**1,379**		**9**	**46**	**1,457**	**153**
New England		**150**			**5**	**74**	
Maine		18				14	
New Hampshire		[2] 9				6	
Vermont		6				3	
Massachusetts		99			5	[2] 26	
Rhode Island		3				8	
Connecticut		15				17	
Middle Atlantic	**2**	**371**			**7**	**110**	**9**
New York		[2] 265			4	53	7
New Jersey	2	22				33	
Pennsylvania		84			3	24	2
East North Central	**1**	**385**			**2**	**195**	**24**
Ohio		104			1	38	5
Indiana	1	15				11	11
Illinois		46				29	1
Michigan		182			1	66	7
Wisconsin		38				51	
West North Central	**1**	**63**		**1**		**71**	**38**
Minnesota	1	16				8	3
Iowa		9				16	17
Missouri		16		1		7	17
North Dakota		4				14	
South Dakota		3				1	
Nebraska		1					
Kansas		14				25	1
South Atlantic	**6**	**72**		**1**	**16**	**166**	**21**
Delaware		2				1	
Maryland	2	22			1	6	
District of Columbia		6				8	
Virginia	1	6			1	72	1
West Virginia	1	12					1
North Carolina	2	19			1	29	
South Carolina					1	3	11
Georgia		3		1	12	29	8
Florida		[2] 2				18	
East South Central	**1**	**34**			**2**	**101**	**19**
Kentucky		19			1	22	5
Tennessee	1	10			1	10	6
Alabama		1				41	7
Mississippi		4				28	1
West South Central	**1**	**45**		**6**	**5**	**531**	**33**
Arkansas		2		2	1	52	5
Louisiana		5			2	6	
Oklahoma	1	9		1		30	1
Texas		29		3	2	443	27
Mountain	**3**	**32**			**4**	**127**	**9**
Montana		2				7	
Idaho	2	8			1	5	
Wyoming	1				1	2	9
Colorado		5			1	28	
New Mexico		1			1	14	
Arizona		4				62	
Utah		[3] 12				8	
Nevada						1	
Pacific		**227**		**1**	**5**	**82**	
Washington		24				24	
Oregon		10			1	8	
California		[3] 193		1	4	50	
Alaska							
Hawaii					1		

[1] Including cases reported as salmonellosis. [2] Including cases reported as streptococcal sore throat.

FOREIGN REPORTS

CANADA

Reported Cases of Certain Diseases—Week Ended May 12, 1951

Disease	Total	Newfoundland	Prince Edward Island	Nova Scotia	New Brunswick	Quebec	Ontario	Manitoba	Saskatchewan	Alberta	British Columbia
Chickenpox	1,144			30		216	582	46	45	82	143
Diphtheria	2					2					
Dysentery:											
Amebic	2									2	
Bacillary	7						2				5
German measles	393	2		1		64	175	3	22	40	86
Influenza	37			16	6		2	10			3
Measles	1,519	1		95	6	416	553	79	12	241	116
Meningitis, meningococcal	2				1		1				
Mumps	772	1		20	1	251	250	20	35	75	119
Scarlet fever	302			1	3	86	46	34	17	46	69
Tuberculosis (all forms)	259	10		1	14	79	33	58	14	16	34
Typhoid and paratyphoid fever	9					5					4
Venereal diseases:											
Gonorrhea	266	5		6	9	62	45	21	17	34	67
Syphilis	90			3	4	35	20	7	6	6	9
Primary	7					4	3				
Secondary	2			1		1					
Other	81			2	4	30	17	7	6	6	9
Whooping cough	173	1				42	85	12		16	17

NORWAY

Reported Cases of Certain Diseases—March 1951

Disease	Cases	Disease	Cases
Diphtheria	20	Pneumonia (all forms)	2,986
Dysentery, unspecified	3	Poliomyelitis	17
Encephalitis, infectious	1	Rheumatic fever	101
Erysipelas	273	Scabies	688
Gastroenteritis	2,388	Scarlet fever	140
Hepatitis, infectious	65	Tuberculosis (all forms)	304
Impetigo contagiosa	1,426	Venereal diseases:	
Influenza	3,685	Gonorrhea	145
Malaria	2	Syphilis	51
Measles	2,933	Other forms	8
Meningitis, meningococcal	12	Weils disease	2
Mumps	102	Whooping cough	1

NEW ZEALAND

Reported Cases of Certain Diseases and Deaths—4 Weeks Ended Apr. 28, 1951

Disease	Cases	Deaths	Disease	Cases	Deaths
Actinomycosis	1		Malaria	3	
Brucellosis	5		Meningitis, meningococcal	8	1
Diphtheria	11	1	Poliomyelitis	7	1
Dysentery:			Puerperal fever	1	
Amebic	8		Scarlet fever	92	
Bacillary	18		Tetanus	3	1
Encephalitis, infectious	3		Trachoma	1	
Erysipelas	14		Tuberculosis (all forms)	143	30
Food poisoning	16		Typhoid fever	6	
Influenza	2				

REPORTS OF CHOLERA, PLAGUE, SMALLPOX, TYPHUS FEVER, AND YELLOW FEVER RECEIVED DURING THE CURRENT WEEK

The following reports include only items of unusual incidence or of special interest and the occurrence of these diseases, except yellow fever, in localities which had not recently reported cases. All reports of yellow fever are published currently. A table showing the accumulated figures for these diseases for the year to date is published in the PUBLIC HEALTH REPORTS for the last Friday in each month.

Plague

Belgian Congo. During the period May 24–29, 1951, plague was reported in Stanleyville Province as follows: Muhono one case, Langbe three, and Bunyo one.

Indochina. For the week ended May 19, 1951, seven cases of plague were reported in Cambodia.

Smallpox

Belgian Congo. During the week ended May 19, 1951, 82 cases of smallpox were reported as compared with 60 for the previous week.

British East Africa. An increase has been noted in the incidence of smallpox in Tanganyika, from 3 cases for the week ended April 7, 1951, the number rose to 17 for the following week.

Cameroon (French). During the period May 1–10, 1951, 32 cases of smallpox were reported compared with 12 for the previous 10-day period.

Ceylon. The recent outbreak of smallpox in Ceylon has subsided with only 3 cases being reported for the week ended May 26, 1951, compared with 26 for the previous week.

Ethiopia. Three cases of smallpox were reported for the week ended April 21, 1951. These are the first cases since the week ended January 13, when one case was reported.

Indochina. The incidence of smallpox in two ports of Vietnam for the week ended May 26, 1951, decreased to about half the number reported for the previous week. In Haiphong the number of cases decreased from 105 to 52 and in Hanoi, from 77 to 44.

Netherlands. During the week ended May 19, 1951, two cases of smallpox were reported in Tilburg where the recent outbreak occurred.

Typhus Fever

Afghanistan. During the month of April 1951, 111 cases of typhus fever were reported in Afghanistan.

Egypt. One case of typhus fever was reported in Cairo for the week ended May 19, 1951.

Eritrea. Three cases of typhus fever were reported in Eritrea for the week ended May 19, 1951.

Turkey. During the week ended May 26, 1951, seven cases of typhus fever were reported compared with five for the previous week.

Brazil. In connection with the recent outbreak of jungle yellow fever in Goiaz State confirmed deaths were reported in counties as follows: Goiaz, March 20, one case; and Jaragua, Mardh 24, one.

Ecuador. One confirmed death, pertaining to the recent outbreak of jungle yellow fever, was reported on April 26, 1951, in Esmeraldas Province, District of Aguas Frias.

Gold Coast. During the period February 25–March 1, 1951, two cases (one death) of yellow fever were reported in Akwatia. Both patients were admitted to the hospital at Kade.

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It contains (1) current information regarding the incidence and geographic distribution of communicable diseases in the United States, insofar as data are obtainable, and of cholera, plague, smallpox, typhus fever, yellow fever, and other important communicable diseases throughout the world; (2) articles relating to the cause, prevention, and control of disease; (3) other pertinent information regarding sanitation and the conservation of the public health.

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